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March 29, 1985

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Re: Research Progress and Forecast Report for Grant AFOSR-84-0331

Dear Dr. Berry:

Since it is already close to the end of March and I have not yet received the forms you told me about over the telephone, I have decided to follow the information included in section 2-C of the brochure of the USAF Administration for Basic Research, and summarize our progress and forecast in the form of a letter addressed to you. In the event that you find that not all the required information has been provided, or that not enough details have been included, please let me know and I shall gladly fullfill all your requests.

This report covers studies carried out in my laboratory during the period of September 1984 through March, 1985, on the project entitled "Role of Protein Phosphorylation in the Regulation of Neuronal Sensitivity". As detailed in my letter to you dated May, 1984, the original research plan submitted to the AFOSR has been revised to conform with the approved budget. Accordingly, our studies on this project focus on three principal topics:

A. Establishing differentiated NG108-15 cells (NG cells) grown in culture as a model system for studying the role of protein phosphorylation in the regulation of neuronal function and neuronal adaptation.

B. Characterizing ecto-protein kinase activity and its endogenous substrates in neural cells, and determining their role in regulating receptor sensitivity.

C. Raising monoclonal antibodies against specific neuronal phosphoproteins, with emphasis on the 54KDa substrate of a GTP-preferring protein kinase, and the substrates of ectokinase activity. These antibodies will be used in experiments designed to provide direct evidence for the function of these phosphoproteins. (AW)

As requested, I provide here a concise report of the progress made in each of these studies during the period reported upon.

A. NG-cells as a model for studying the role of neuronal phosphoproteins. When neural cells of the clone NG108-15 are treated for several days with agents that increase the levels of intracellular cyclic AMP, they undergo morphological, physiological and biochemical differentiation, including the ability to form functional synapses. Differentiated NG cells provide, therefore, an optimal model system of a homogenous cell population most suitable for detailed biochemical investigation of the role of specific phosphoproteins in neural function. In spite of the large body of evidence in the literature on the suitability of this model system for investigating various neuronal functions, studies on the protein phosphorylation systems of these cells have not been reported yet. Our first goal in this line of investigation has been to document and characterize the endogenous protein phosphorylation systems of these cells. This goal has now been achieved. The emphasis in these studies has been on identification of the phosphorylation systems in differentiated cells and determination of their uniqueness compared to the systems in proliferating cells. This was done by measuring the endogenous phosphorylation of specific proteins in various subcellular fractions from cells treated for 6 days with 1mM dibutyryl cyclic AMP, in comparison with corresponding fractions from non-treated cells. Bi-directional changes, namely, both increases and decreases in the phosphorylation of specific proteins, have been documented in differentiated vs. proliferating NG cells. The major changes occurred in preparations of purified plasma membranes. The endogenous phosphorylation in plasma membranes of proliferating NG cells is very low and resembles that of non-excitable cells. In contrast, plasma membranes purified from differentiated NG cells exhibit a very high level of phosphorylative activity towards a multiplicity of endogenous protein substrates, and has a pattern resembling that obtained with brain membranes. Furthermore, phosphorylation of a 54KDa protein by GTP with properties such as seen in synaptic membranes is induced in NG cells by the differentiating treatment. These studies have been carried out by a graduate student, Mr. T. Davis, under my supervision. The findings have been presented at the 10th annual meeting of the Society for Neuroscience (with credit given to the AFOSR grant). Mr. Davis is currently writing a thesis based on these studies and this will form the basis for preparing a manuscript to be submitted for publication in the Journal of Neurochemistry.

B. Ecto-protein kinase and specific substrates on the external surface of neural cells. It is well known that extracellular ATP exerts potent effects on the activity of plasma membranes in excitable cells. Moreover, ATP is co-released with neurotransmitters upon stimulation, and can then interact with components on the external surface of the plasma membrane. Some of these effects may be mediated by a protein kinase localized on the surface of neuronal cells (an ecto-kinase), utilizing extra-

cellular ATP to phosphorylate specific proteins in the plasma membranes. Due to the heterogeneity and lack of full integrity of synaptosomes from brain, direct evidence for this activity can not be obtained by studying synaptosomal preparation. Based on the studies supported by the AFOSR grant, we now have direct evidence that cells of neural origin have ectokinase and specific protein substrates for its activity. These studies have been presented at the 16th meeting of the American Society for Neurochemistry in a poster which you have seen. In brief, neuroblastoma x glioma hybrid cells of the clone NG108-15 (NG cells) were grown in a chemically defined, serum-free medium in individual wells of 96-multiwell plates. Ectokinase assays have been performed with cells attached to the bottom of the well, covered with 80ul of a modified Krebs-Ringer buffer (containing 0.8mM $MgCl_2$ and 1.8mM $CaCl_2$). Reactions were initiated by adding γ-^{32}P-ATP (2uM; 10uCi/well). Intact NG cells utilized extracellular AT^{32}P to phosphorylate endogenous proteins, as well as exogenous proteins (casein) added to the medium. This activity could be eliminated by mild pretreatment of the cell surface with low (1-10ug) trypsin. The time-course of phosphorylation of endogenous proteins by intracellular kinases (monitored in cells labeled with ^{32}Pi) was different (slower) from that of the ectokinase activity. Further, different protein components are phosphorylated by these two activities. The major protein substrates of ectokinase in NG cells have apparent M.w.'s of 190K, 120K, 97K and 54K. Their phosphorylation by extracellular AT^{32}P showed selective sensitivity to Mn^{++}-ions, Ca^{++}-ions concentration and the calcium channel blocker verapamil (1uM). Correlative data suggest that ectokinase activity may play a role in the regulation of calcium uptake by intact neural cells. We have found that extracellular ATP stimulates $^{45}Ca^{++}$-uptake in NG cells with a $K_{0.5}$ for ATP that is almost identical to the Km of the ectokinase for extracellular ATP. I am currently preparing this study for submission to a journal and will send you a pre-publication copy of the paper.

C. Preparation of monoclonal-antibodies to specific neuronal phosphoproteins. These studies are carried out in collaboration with Dr. E. Kornecki, head of a Monoclonal Antibody Core in our department. During the period covered by this report, we have developed a rapid method for screening hybridomas which secrete antibodies that interact specifically with substrates of protein kinase activity, and began in the preparation of monoclonal antibodies to the 54KDa protein substrate of a GTP-preferring kinase in neuronal membranes.

Specific antibodies directed against a single protein can be obtained by the hybridoma technique even when using in the immunization a crude preparation in which the antigen of interest is only a minor protein component. However, the success of such an approach depends on the availability of a reliable, selective and rapid screening method for the detection of hybrid cell colonies secreting the desired antibody. Dr. E. Kornecki has immunized mice with a Triton extract of calf-brain membranes and obtained antisera that were positive in an ELISA assay against the immuni-

zing antigen at a 1.10,000 serum dilution. Three of these antisera immunoprecipitated that 54K protein phosphorylated by $GT^{32}P$ (tested by Protein-A immunoprecipitation followed by SDS-gel electrophoresis and autoradiography). These antisera were used in developing a radioimmunoassay (RIA) for detecting antibodies directed against substrates of protein kinase activity. Triton extract was incubated with $GT^{32}P$ and Mn^{++} for 4 min at 30°C, then chilled to 0°C, incubated with antiserum overnight at 4°C, then treated with denatured Staph A (Pansorbin) and the immunoprecipitate was washed and counted. Controls with buffer alone and nonimmune serum and immune serum all gave about the same counts. This was found to be due to nonspecific interactions in the controls (determined by gel electrophoresis). The problem of high background was solved by using a desalting column. After the reaction of Triton extract with Mn-$GT^{32}P$, 10mM NaF was added (to minimize phosphatase activity) and the protein was separated from ^{32}Pi(free) by chromotography through 10ml bed volume of Biogel-P6. The fraction containing ^{32}P-bound proteins was used as antigen in the immunoprecipitation phase of the assay. In a typical RIA using this antigen, the Prot. A pellet after buffer blank gave 824 CPM, nonimmune serum gave 858 CPM, immune serum gave 2,590 CPM and immune-serum diluted 1:10 gave 3,289 CPM (a potent antibody). Gel electrophoresis verified that only the precipitate from the immune serum contained phosphorylated 54KDa protein. Spleen cells of mouse #4 were used in a fusion protocol. Screening was carried out using the ^{32}P-RIA as described above, except that both $AT^{32}P$-labeled and $GT^{32}P$-labeled Triton extract were used. Gel electrophoresis verified that also after P6-column chromatography of reaction carried out with $GT^{32}P$, the 54k is the major phosphorylated protein product (over 60% of total radioactivity), while after reaction with $AT^{32}P$ the 54k contained less than 10% of total radioactivity. The Staph-A used in the screening was conjugated with goat anti-mouse IgG prior to the immunoprecipitation. Using this screening assay we have detected 4 hybridoma colonies producing supernates that gave positive RIA reading with Triton extract phosophorylated with $GT^{32}P$ but not with $AT^{32}P$. After propagation and cloning of these hybridomas, we shall determine by gel electrophoresis which produce antibodies that immunoprecipitate ^{32}P-54KDa proteins, studies on the function of this specific phosphorylation system will then be initiated.

D. Summary and Forecast

We believe that substantial progress has been made in all the lines of investigation pursued as part of this project. In each of these areas, the necessary ground work has almost been completed. We have characterized the endogenous phosphorylation systems of differentiated NG cells, obtained direct evidence for the activity of ecto-protein kinase in these cells, and have developed conditions for preparring and screening monoclonal antibodies to specific neuronal phosphoprotein. With this data base at hand, we can now initiate the studie designed to determine the role of specific phosphoproteins in the regulation of neuronal sensitivity to external stimuli, as outlined in our

proposal. We anticipate now only one deviation from the approaches we have planned originally. A substantial progress has been made during the last year in developing conditions for growing in culture pure neuronal population of primary cells obtained from the central nervous system. Dr. Samuel Weiss will complete, in September, 1985, a two-year post-doctoral fellowship at the laboratory of Dr. Joel Bockaret in France, where he has been intimately involved in the development of these procedures. In addition, he is proficient in the methodology for measuring receptor activity and regulation. Dr. Weiss has accepted my offer to join us as a Research Associate to work on the project supported by the AFOSR. For this purpose, we have delayed utilization of approximately $15,000 to $20.000 of the funds allocated for year 01 to year 02 of the award. The Office of Sponsored Projects (OSP) at the University of Vermont has informed me yesterday that as of the beginning of this month, we are operating with a combined budget for a two-year period; the total amount has been changed and a new expiration date of August 31, 1986 has been set. I hope that this interpretation of the OSP meets with your approval. I anticipate, therefore, an accelerated progress during the subsequent years of this award and believe that the research efforts of an advanced investigator, instead of a beginning post-doc as originally planned, will further ennance the accomplishment of our scientific goals.

In closing, I wish to thank you again for your efforts on our behalf and to express my hopes that you will find our progress meeting with your expectations.

Yours sincerely,

Yigal H. Ehrlich, Ph.D.
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Depts. of Psychiatry and
Biochemistry

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